AD-A285 216

ATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1994	Journal article

4. TITLE AND SUBTITLE
Colonization of chicks by non-culturable campylobacter spp.

5. FUNDING NUMBERS
PE - n.a.
PR -
TA -
WU -

6. AUTHOR(S)
Stern NJ; Jones DM; Wesley IV; Rollins DM

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

8. PERFORMING ORGANIZATION REPORT NUMBER
NMRI 94-38

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Medical Research and Development Command
National Naval Medical Center
Building 1, Tower 12
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5606

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
N.A.

11. SUPPLEMENTARY NOTES
Reprinted from: Letters in Applied Microbiology 1994 Vol.18 pp. 333-336

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS
food-borne bacteria; enteric diseases; campylobacter jejuni; campylobacter coli; bacterial colonization of chickens

15. NUMBER OF PAGES
4

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Letters in Applied Microbiology 1994, 18, 333–336

Colonization of chicks by non-culturable *Campylobacter* spp.

N.J. Stern, D.M. Jones[1], I.V. Wesley[2] and D.M. Rollins[3]

Poultry Microbiological Safety Research Unit, Richard Russell Agricultural Research Centre, US Department of Agriculture-Agricultural Research Service, Athens, GA, USA, [1]Public Health Laboratory Service, Manchester, UK, [2]USDA-ARS, National Animal Disease Center, Ames, IA, USA, and [3]Navy Medical Research Institute, Bethesda, MD, USA

FS/192: accepted 7 January 1994

N.J. STERN, D.M. JONES, I.V. WESLEY AND D.M. ROLLINS. 1994. Six suspensions of non-culturable *Campylobacter* spp. were administered by gavage to day-of-hatch chicks. Four non-culturable isolates of *Campylobacter* spp. were found to colonize low numbers (5/79) of 1-week-old chicks, while two isolates did not (0/30). The original and recovered *Campylobacter* spp. isolates were serotyped and examined by restriction enzyme analysis. Evidence of clonality of two *Camp. jejuni* isolates was demonstrated.

INTRODUCTION

Campylobacter jejuni/coli is recognized as a major cause of intestinal infections in humans. These infections are often transmitted through foods of animal origin, with undercooked or mishandled poultry frequently implicated as the vehicle of numerous sporadic cases (Deming *et al.* 1987). Consequently, we are interested in determining ways to diminish the numbers of the organism associated with poultry.

Accurate epidemiologic assessment for the transmission of the organism to broiler chickens is needed to control *Campylobacter* spp. association with poultry. Thus far, the literature has suggested numerous possible reservoirs for the organism, none of which are consistently implicated. The existence of a non-culturable form of the organism has been suggested (Rollins and Colwell 1986) but conclusive evidence for transmission of this bacteria form to chickens has been lacking. The subject of this report serves to confirm that *Campylobacter* spp., although not detectable, may be capable of colonizing chickens.

MATERIALS AND METHODS

Bacteriology

Six isolates of *Campylobacter* spp. of poultry origin were grown individually on Brucella-FBP agar overnight (42°C, microaerobic atmosphere), and the growth suspended in individual flasks containing 500 ml of phosphate buffered saline (PBS; pH 7·2) at *ca* $10^{8·5}$ cells ml^{-1}. Cultures were held in parafilmed, screw-capped media bottles at 4°C. Suspensions were enumerated by spread plating on Brucella-FBP agar, initially, and weekly thereafter. When the organisms were no longer detectable by direct plating (4–7 weeks), 50 ml of the original suspensions (OS) were inoculated into selective enrichment (Stern and Line 1992) and 50 ml inoculated again with the OS 2 d later. Just prior to challenging chicks, 200 ml of the OS was centrifuged (8000 *g*, 5 min) and resuspended in 15 ml of PBS, with 5 ml being tested again by enrichment methodology. Portions of this suspension were used to challenge chicks as described below.

Chickens

Day-of-hatch chicks were transported from a commercial hatchery to our laboratory where they were placed in raised wire floor isolation units (IUs) ventilated with positive pressure filtered air. The paper pads on which the birds were transported were selectively enriched to demonstrate absence of culturable *Campylobacter* spp. Eleven to 20 chicks were placed in each IU, provided feed and water *ad libitum*, and maintained at 95°F (*ca* 35°C) with constant lighting throughout the experiments. The birds were allowed 24 h to acclimate to the IUs before experimental challenges were carried out. These chicks were gavaged with 0·2 ml of the above described suspension. At 7 d of age, birds were sacrificed by cervical dislocation, ceca were aseptically removed, diluted 1 : 3 in PBS, and streaked onto Campy-Cefex medium (Stern *et al.* 1992). Plates were incubated for 24 h at 42°C, under microaerobic atmosphere, before examining colonial morphology and viewing under

Correspondence to: Dr Norman J. Stern, Poultry Microbiological Safety Research Unit, Richard Russell Agricultural Research Center, US Department of Agriculture-Agricultural Research Service, PO Box 5677, Athens, GA 30613, USA.

phase-contrast microscopy for the presence of typical *Campylobacter* spp.

Serotyping

Both the original (O) and the recovered (R) *Campylobacter* spp. strains were coded and sent to cooperating laboratories. One laboratory employed the heat-stable serotyping scheme described by Penner and Hennessey (1980), and both facilities used the heat-labile serotyping scheme described by Lior *et al.* (1982).

Restriction enzyme analysis

Bacterial isolates were cloned three times on brain heart infusion agar containing 10% defibrinated bovine blood. Colonies were harvested, resuspended in 0·85% NaCl or in 0·1 mol l^{-1} phosphate buffered saline, and pelleted by centrifugation (8000 *g*, 30 min). The resultant pellet was resuspended in 0·5 ml of buffer (10 mmol l^{-1} TRIS, 1 mmol l^{-1} EDTA (pH 8·0); TE buffer) with 25% sucrose and was frozen (−10°C) until the time of DNA extraction. High molecular weight DNA was recovered by equilibrium centrifugation (416 000 *g*, 3 h, 15°C) in CsCl (1·25 g ml^{-1} of 50 mmol l^{-1} TRIS, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} NaCl). The viscous band was extracted from the side of the centrifuge tube through a 16-gauge needle. The resultant DNA was dialysed extensively against TE buffer. The DNA concentration was determined spectrophotometrically ($O.D._{260}$). The DNA (2 μg) was digested (3–4 h, 37°C), with *Bgl*II or *Hha*I in a 20 μl reaction mixture in buffers supplied by the manufacturer. After digestion, 5 μl of tracking dye (0·1% bromphenol blue, 20% Ficoll type 400) was added to each sample, and DNA fragments were separated on 1·0% agarose (16 h, 60 V) in a horizontal gel bed (120 × 25 cm). At the completion of electrophoresis, gels were stained (1 h) with ethidium bromide (0–25 mg ml^{-1}), visualized with short-wave u.v. light and photographed, using a red filter.

RESULTS AND DISCUSSION

Viability of strains of *Campylobacter* spp. held at 4°C diminished and became non-culturable over 8 weeks (Table 1). Sixty per cent of each bacterial suspension was used to verify a lack of proliferating capacity prior to chicken challenge and no organisms were recovered. Chick challenges with these non-culturable *Campylobacter* spp. suspensions resulted in low numbers of the birds becoming colonized (Table 1).

Laboratory No. 1 had no matches and Laboratory No. 2 had three of the four O and R pairs of *Campylobacter* spp. matched for the heat-labile serotyping analysis. Heat-stable

Table 1 Colonization of chicks by non-culturable *Campylobacter* spp.*†

Strain identification	Species identity	Weeks at 4°C before non-culturability	No. of chicks colonized per no. of chicks tested	Serotyping results: Laboratory No. 1 O-somatic‡	Laboratory No. 1 Heat-labile§	Laboratory No. 2 Heat-labile§	Restriction analysis results
Colonizing, non-culturable strains:							
RTA38-O	*coli*	4	1/20	28	8	8, 29	Differed at
RTA38-R	*coli*			30, 40	29	8, 29	high mol. wts
814-9-O	*jejuni*	4	1/19	4, 16	Rough	17	Same
814-9-R	*jejuni*			4, 3, 16w	NT	17	patterns
5C-O	*jejuni*	4	1/20	23, 36	NT	NT	Same
5C-R	*jejuni*			23, 36	NT	NT	patterns
CCD20-O	*coli*	5	2/20	NT	29	17	Different
CCD20-R	*coli*			51	45	17	patterns
Non-colonizing, non-culturable strains:							
CSJ-3-O	—	3	0/19	NC	NC	NC	NC
XPTA12-O	*jejuni*	8	0/11	NC	NC	NC	NC
Unchallenged chicks	—	—	0/18	NC	NC	NC	NC

* -O is the original strain, -R is the recovered isolate.

† NC, No comparisons; NT, non-typable.

‡ Passive haemagglutination assay of Penner and Hennessey (1980).

§ Slide agglutination assay of Lior *et al.* (1982).

(somatic) serotyping analysis matched one and partially matched another of the four pairs (Table 1). Restriction enzyme analysis with the endonucleases *Bgl*II and *Hha*I indicated that at least two of the pairs (814-9 and 5C) were identical and two pairs were dissimilar (RTA38 and CCD20) with respect to the O and R strains (Fig. 1). This is most clearly evident in *Hha*I digests (Fig. 1b). One explanation of the data is that genetic and antigenic change may occur to give differences in O and R paired strains in these assays.

For each pair of *Camp. jejuni* isolates no change in heat-stable antigen patterns and restriction enzyme profiles (Fig. 1, lanes 3, 4 and 5, 6) was noted between the O and the R isolates. In contrast, for *Camp. coli* strains differences in heat-stable antigen profiles and in restriction enzyme pattern were noted between the O and the corresponding R strain (Fig. 1, lanes 1, 2 and 7, 8).

Restriction enzyme analysis is a sensitive technique based on the relative stability of chromosomal DNA. In contrast, the phenotypic expression of flagellar proteins is variable and may underlie the observed serotyping differences in heat-labile patterns. Genetic rearrangement associated with flagellar antigenic variation has been documented for *Camp. coli* (Guerry *et al.* 1988). That *Camp. coli* paired strains CCD20-O and CCD20-R differed in three of four assays suggests that genetic and antigenic change may have occurred. Alterations in restriction enzyme profiles and antigenic profiles in *Campylobacter* spp. recovered from experimental animals when compared to the original infecting strain have been documented in cattle (Corbeil *et al.* 1975; Wesley and Bryner 1989). Thus, variations seen in the *Camp. coli* paired strains may represent a similar event in birds. Another possibility was that *Campylobacter* spp. came from the hatchery into our IUs, and these were not detected during initial screening or were not detected within the IUs containing the control chicks.

Some controversy exists regarding capacity of non-culturable *Campylobacter* spp. to colonize chicks. Medema *et al.* (1992) reported that non-culturable *Camp. jejuni* used in their study were not able to colonize chicks. They prepared their viable/non-culturable *Campylobacter* spp. at 20–30°C, where dormancy to death is much more rapid than at refrigeration temperatures. Saha *et al.* (1991) reported that non-culturable *Camp. jejuni* could be converted to a resuscitated form after passage through rat gut. Jones *et al.* (1991) indicated that colonization of mice can be established after challenge by non-culturable forms of *Camp. jejuni*. Prior to the present publication, no conclusive evidence had been presented to demonstrate that at least some non-culturable forms of *Campylobacter* spp. were capable of colonizing chicks. The presence of a colonizing but non-culturable form of *Campylobacter* spp. provides one explanation for transmission of the bacterium to broilers. After a single bird initiates colonization, secondary infection could serve to colonize other chickens (Stern *et al.* 1988). *Campylobacter* spp. could pass from within warm blooded hosts

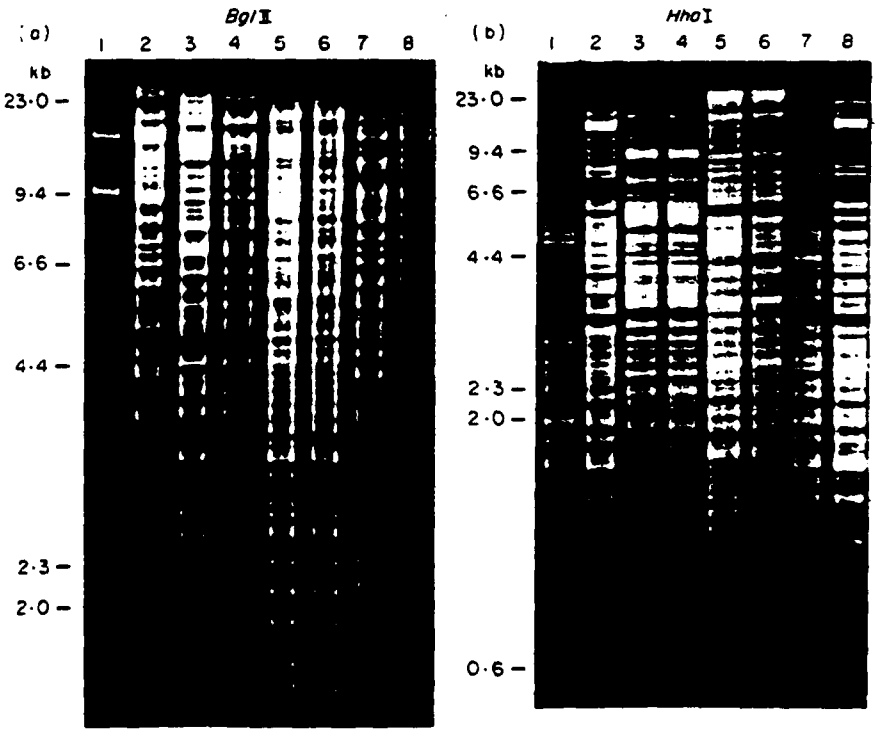

Fig. 1 Restriction enzyme analysis of original (-O) and recovered (-R; colonizing but non-culturable) *Campylobacter* spp. employing *Bgl*II (a) and *Hha*I (b) restriction enzyme digest analysis. Lane 1, RTA38-O; lane 2, RTA38-R; lane 3, 814-9-O; lane 4, 814-9-R; lane 5, 5C-O; lane 6, 5C-R; lane 7, CCD20-O; lane 8, CCD20-R

into a hostile, exterior environment. To survive, these bacteria must be able to adapt to endure such exposure prior to colonizing its next warm blooded host. Such an adaptation confers survival of the genus and potentially accounts for the transmission of the organism to flocks of poultry.

ACKNOWLEDGEMENT

The authors thank and acknowledge the contributions of C. Patton, Centers for Disease Control, Atlanta, Georgia, for serotyping analysis.

REFERENCES

Corbeil, L.B., Schurig, G.G.D., Bier, P.J. and Winter, A.J. (1975) Bovine venereal vibriosis: antigenic variation of the bacterium during infection. *Infection and Immunity* 11, 240–244.

Deming, M.S., Tauxe, R.V., Blake, P.A., Dixon, S.E., Fowler, B.S., Jones, T.S., Lochamy, E.A., Patton, C.M. and Sikes, R.O. (1987) *Campylobacter* enteritis at a university: transmission from eating chicken and from cats. *American Journal of Epidemiology* 126, 526–534.

Guerry, P., Logan, S.M. and Trust, T.J. (1988) Genomic rearrangements associated with antigenic variation in *Campylobacter coli*. *Journal of Bacteriology* 170, 316–319

Jones, E.M., Sutcliffe, E.M. and Curry, A. (1991) Recovery of viable but non-culturable *Campylobacter jejuni*. *Journal of General Microbiology* 137, 2477–2482.

Lior, H., Woodward, D.L., Edgar, J.A., Laroche, L.J. and Gill, P. (1982) Serotyping *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. *Journal of Clinical Microbiology* 15, 761–768.

Medema, G.J., Schets, F.M., van de Giessen, A.W. and Havelaar, A.H. (1992) Lack of colonization of 1-d-old chicks by viable, non-culturable *Campylobacter jejuni*. *Journal of Applied Bacteriology* 72, 512–516.

Penner, J.L. and Hennessey, J.N. (1980) Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat stable antigens. *Journal of Clinical Microbiology* 12, 732–735.

Rollins, D.M. and Colwell, R.R. (1986) Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in natural aquatic environments. *Applied and Environmental Microbiology* 52, 531–538.

Saha, S.K., Saha, S. and Sanyal, S.C. (1991) Recovery of injured *Campylobacter jejuni* cells after animal passage. *Applied and Environmental Microbiology* 57, 3388–3389.

Stern, N.J. and Line, J.E. (1992) Comparison of three methods for recovery of *Campylobacter* spp. from broiler carcasses. *Journal of Food Protection* 55, 663–666.

Stern, N.J., Stan Bailey, J., Blankenship, L.C., Cox, N.A. and McHan, F. (1988) Colonization characteristics of *Campylobacter jejuni* in chick ceca. *Avian Diseases* 32, 330–334.

Stern, N.J., Wojton, B. and Kwiatek, K. (1992) A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. *Journal of Food Protection* 55, 514–517.

Wesley, I. and Bryner, J.H. (1989) Antigenic and restriction enzyme analysis of isolates of *Campylobacter fetus* subsp. *venerealis* recovered from persistently infected cattle. *American Journal of Veterinary Research* 50, 807–813.